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Rates of Transformation of Methyl Parathion and Diethyl Phthalate by Aufwuchs Microorganisms

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16. ABSTRACT

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Rates of Transformation of Methyl Parathion and Diethyl Phthalate by Aufwuchs Microorganisms

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Using batch cultures, we determined transformation rates for low concentrations of two toxicants—an insecticide, methyl parathion (*O,O*-dimethyl *O*-*p*-nitrophenyl phosphorothioate), and a plasticizer, diethyl phthalate—by aufwuchs, aquatic microbial growth attached to submerged surfaces or suspended in streamers or mats. Aufwuchs samples were collected from field sites, an indoor channel, and a continuous-flow fermentor. Aufwuchs fungi, protozoa, and algae did not transform methyl parathion or diethyl phthalate, but bacteria rapidly transformed both chemicals. Second-order transformation rate coefficients, K_b, based on total plate counts of bacteria in aufwuchs, were determined for potential use in a mathematical model capable of predicting the transport and fate of chemicals in aquatic systems. K_b for both methyl parathion and diethyl phthalate decreased as the concentration of total bacteria, [B], increased in aufwuchs. This effect resulted from the proportion of nontransformer to transformer bacteria increasing as [B] increased and from the rate of transformation per transformer cell decreasing as [B] increased. First-order transformation rate coefficients, K_1, were relatively stable per unit of surface area colonized by aufwuchs, because K_b decreased as [B] increased ($K_1 = K_b \times [B]$).

Mathematical models capable of accurately predicting the transport and fate of chemicals, especially toxic substances, in aquatic environments are being developed by the U.S. Environmental Protection Agency. These transport and fate models incorporate rate coefficients of environmental processes to predict the movement of chemicals among various compartments of ecosystems, including water, sediment, biota, and air. Rate coefficients for chemical hydrolysis, photolysis, sorption, volatilization, and some other environmental processes can be readily determined in the laboratory. Determining accurate rate coefficients for microbial transformations of chemicals that will have general application in field situations, however, is not a matter that has been resolved with our current knowledge of such processes. Much basic information needs to be obtained as to what kinds of microorganisms under what circumstances will transform a chemical, what range of transformation rates will occur, and what factors influence the presence and activities of microbial populations capable of transforming certain chemicals.

Microorganisms are distributed in three phases in the aquatic ecosystem: suspended in water, associated with aufwuchs (microbial growth attached to submerged surfaces or suspended in streamers or mats), and associated with sediments. Paris et al. (4) have reported rates of transformation of xenobiotic chemicals by suspended bacteria. Using a second-order rate expression, Paris and co-workers observed reproducible rate coefficients for transformations of 2,4-D butoxyethyl ester, malathion, and chloropropham by suspended bacteria.

In our laboratory ecosystems, aufwuchs were the predominant microbial constituent, and we investigated the utility of second-order rate coefficients, K_b, for transformations of chemicals by aufwuchs communities.

The specific objectives of the investigations reported here included: (i) the determination of whether all or only part of the loss of methyl parathion (MP), *O,O*-dimethyl *O*-*p*-nitrophenyl phosphorothioate, and diethyl phthalate (DEP) in aufwuchs resulted from microbial transformations of the chemicals; (ii) the determination of which group(s) of microorganisms was responsible for transformations of MP and DEP in aufwuchs; (iii) the determination of the range of variation in K_b in aufwuchs, based on estimates of populations of the group(s) of microorganisms responsible for the transformations; and (iv) the determination of the major cause(s) of large variations which may occur in the transformation rate coefficients in aufwuchs.

Methyl parathion was chosen as a toxicant because concentrations in the microgram-per-liter range can be determined by using standard gas-liquid chromatography techniques and be-

cause some microbial transformation rate coefficients are already published (6). Diethyl phthalate was used as a second toxicant so that MP results could be compared with those of a compound of dissimilar structure. Standard plate counts were chosen to indicate bacterial population densities used in calculating rate coefficients for toxicant transformations. The limitations of plate counts as a quantitative procedure are summarized by Buck (2); however, we currently have no satisfactory alternative procedure.

MATERIALS AND METHODS

Sources of microorganisms. The large, flowing-water laboratory stream used as one of the sources of microorganisms was a modified form of the aquatic ecosystem simulator (AEcoS), the indoor water channel of the laboratory (5). The modifications included establishing two independnet channels, each containing eight 250-liter tanks. The tanks are called continously stirred tank reactors (CSTRs); water flows successively from tank to tank. Each CSTR contained a Teflon-covered Plexiglas paddlewheel rotating at 4.25 cm/s at the circumference to provide water turbulence and aeration. Both channels were supplied with 500 liters of deionized, ultraviolet-irradiated water per day. The channels were lighted with 2,000 footcandles (2.152×10^4 lx) of fluorescent light on 12-h light-dark cycles. The pH fluctuated between approximately 6.5 and 9.5. CSTRs were inoculated with water from North Georgia lakes and streams to initiate the establishment of microbial communities; nutrient treatments of the CSTRs are shown in Fig. 3. Water temperature was 20°C.

A second source of microorganisms used in our studies was a Microferm fermentor (New Brunswick Scientific Co., Inc.) inoculated in the same manner as the AEcoS and continuously supplied with an inorganic nutrient solution and 10 mg of glycerol per liter. The fermentor was lighted on a 12-h light-dark cycle with fluorescent lights at about 500 footcandles (5.38×10^3 lx); water temperature was 20°C. The nutrient solution was supplied at pH 7, but the pH in the fermentor fluctuated as it did in the AEcoS during light-dark cycles. The fermentor was spiked daily with MP to give a final concentration of about 150 μg/liter.

Aufwuchs samples were also collected from two field sites, Watson Mill State Park and High Shoals, near Athens, Ga. Both of these river sites have large shallow areas from which aufwuchs can be easily collected by being dislodged from submerged rock surfaces.

Gas-liquid chromatography. Concentrations of MP and DEP were determined upon extraction of water samples with 2,2,4-trimethyl pentane (isooctane) according to an earlier procedure (3). Determinations were made by using a Tracor 550 gas-liquid chromatograph equipped with an ^{63}Ni detector and a 2-m column (internal diameter, 4 mm) packed with 3% SE-30 on 80/100-mesh Gas-Chrom Q. Extraction efficiency for MP was 96.9% (standard deviation, ±3.3; n, 10) and for DEP was 97.0% (standard deviation, ±3.6; n, 8). Organic compounds that eluted close to DEP in gas-liquid chromatographic analyses were liberated by blending aufwuchs. These interfering compounds were not a major problem unless the samples were blended for longer than 5 s.

Verification of microbial transformation. Candicidin, used to select for bacterial activity, was supplied by S.B. Penick & Co. Candicidin is toxic to algae, protozoa, and fungi. We have found that 10 to 100 mg of candicidin per liter is useful in selecting for blue-green algae (cyanobacteria) in mixed algal cultures, and 500 mg/liter is useful for toxicity to all organisms other than bacteria.

Incubation of aufwuchs in the dark as opposed to the light, to determine the involvement of algae in the transformation of the toxicants, was accomplished by incubating unblended samples of aufwuchs in 250-ml Erlenmeyer flasks at 20°C in a shaker incubator. By covering the flasks with aluminum foil, we ensured that the aufwuchs were incubated in the dark. The flasks were incubated for 72 h and then spiked to a final concentration of approximately 120 μg of MP per liter or 350 μg of DEP per liter, and then the rates of loss of the toxicants were measured.

Isolation and determination of proportions of transformer bacteria. Bacteria were isolated at 35°C from standard plate counts of blended aufwuchs samples by picking colonies and transferring them to flasks containing a 10-fold dilution of standard methods nutrient broth (Difco Laboratories); 60 colonies per plate were picked. The flask cultures were incubated in the dark at 20°C in a shaker incubator. As each flask culture became slightly turbid with growth, the flasks were spiked to approximately 100 μg of MP per liter or 350 μg of DEP per liter. The cultures were extracted with isooctane 48 h after being spiked, and the concentrations of the toxicants were determined. A culture that showed a loss of 30% or more of MP or DEP was concluded to be a transformer isolate; however, transformer bacteria almost always showed no detectable quantity of MP or DEP in the broth cultures, whereas nontransformer bacteria showed no loss of the toxicant. The percentage of transformers was determined by dividing the number of isolates that transformed the toxicant (× 100) by the total number of colonies picked from a plate. More than 900 bacterial isolates were tested for ability to transform MP or DEP.

Transformer bacteria picked from colonies on standard plate counts and transferred to the dilute broth gave rise to populations which all continued to transform MP or DEP. Likewise, nontransformers isolated and transferred to the dilute broth gave rise to populations none of which transformed MP or DEP. This method of determining transformers detects organisms capable of transforming the chemicals in the presence of other nutrients, rather than just those organisms capable of using the chemicals as sole carbon sources. Bacterial species that transformed MP were identified by Southeast Laboratories, Inc., Atlanta, Ga.

Determinations of rates of transformations. Rate studies were performed by using aufwuchs samples collected from the AEcoS, the fermentor, and field sites. The aufwuchs samples were blended to make a suspension of the aufwuchs-associated bacteria so that they could be diluted, replicate cultures could be made, and bacterial concentrations could be determined. The number of colony-forming units of bacteria

did not increase after blending aufwuchs for 0.5, 1.0, or 1.5 min, showing that the bacteria were thoroughly suspended after 5 s of blending. Blending aufwuchs samples did not affect the rates of transformation of MP and DEP within 8 to 10 h. Bacterial concentrations tended to decrease within 24 h after blending and then increase for the next several days, apparently by utilizing nutrients released from blended aufwuchs. For this reason, transformation rate studies on the blended aufwuchs were completed within a short time, usually within 8 h after blending.

Rate data were obtained from batch cultures of blended aufwuchs in 250-ml Erlenmeyer flasks. Batch cultures for MP experiments were made in duplicate by pipetting 15 ml of blended aufwuchs into the flasks and diluting them up to 180 ml with deionized water. Deionized water, rather than a buffer solution, was used to avoid possible catalytic effects of salts and metal ions as reported by Smith et al. (6). Batch cultures for DEP experiments were made in sets by pipetting 5 ml of blended aufwuchs into one flask, 10 ml into a second flask, and 20 ml into a third flask. The sets were referred to as triplicates because they were subcultures of the same aufwuchs sample. This procedure usually gave a range of one to four half-lives of the toxicant during the rate study. Because the bacterial concentrations varied by about four orders of magnitude, it was difficult to estimate how much aufwuchs should be used to give at least two half-lives of transformation.

Rate data were also obtained by using the continuous-flow fermentor spiked to approximately 150 μg of MP per liter after the flow of water and nutrients had been stopped. Duplicate water samples were pipetted from the fermentor every 1 to 3 h, depending on the rate of transformation, for up to 10 h. Only one half-life of MP could be measured by using this procedure. The water and nutrient flow would have to be shut off for an extended length of time to measure more than one half-life, and we were concerned that nutrient limitation or other factors might affect the rates of transformation if the flow was cut off for longer than about 10 h.

No lag phase in MP or DEP transformation was observed, and the rates of loss were first order with respect to toxicant concentrations in batch cultures and in the fermentor; i.e., the velocity of loss was directly proportional to toxicant concentrations. First-order rate coefficients, K_f, were calculated by using the least-squares method.

K_b values were calculated by dividing K_f by bacterial concentrations. Bacterial samples were taken at the zero hour sampling of MP and DEP, plated in duplicate with standard methods nutrient agar, and incubated at 37°C for 48 h. Incubation at 35°C was preferred over incubation at 20°C because the large quantity of plates required each day by our experiments necessitated as short an incubation time as possible; no difference was found between counts of plates incubated at 35°C and counts of plates incubated at 20°C. To correlate K_b with bacterial concentrations in aufwuch biomass, ash-free, dry-weight determinations (AFDW) of blended aufwuchs were made by using a procedure for periphyton (1).

Effects of carbon sources. Diauxie was tested by using axenic cultures of *Brevibacterium* sp. in 0, 10, 100, and 1,000 mg of glucose, glycerol, and nutrient broth per liter in a basal salts solution spiked to a final concentration of approximately 1 mg of DEP per liter. *Flavobacterium aquatile* was tested in 0, 0.8, and 8.0 g of nutrient broth per liter spiked to a final concentration of approximately 8 μg of MP per liter K_b was estimated by using mean bacterial concentrations during the growth phase.

RESULTS

Verification of microbial transformation. A rapid loss of MP and DEP occurred in batch cultures containing several grams or more of aufwuchs biomass per liter. To determine whether the loss was a result of biological activity and to identify which group(s) of microorganisms was responsible for the loss, we conducted several experiments to elucidate the causes of the observed rapid loss of the toxicants. Biological transformation of the toxicants was indicated because the loss of MP and DEP did not occur in unblended aufwuchs samples that had been autoclaved for 2 h or in aufwuchs that had been blended for 5 s and autoclaved for 15 min. Blending the aufwuchs apparently exposed the organisms responsible for the loss of MP and DEP to the effects of autoclaving.

Incubating aufwuchs in the dark, as opposed to the light, for 72 h did not affect the rates of MP and DEP loss, indicating that the loss was not mediated by algae, which made up the bulk of the aufwuchs biomass. The addition of 500 mg of candicidin per liter, a polyene antibiotic that is toxic to fungi, protozoa, and algae (including blue-green), also did not affect the rates of loss of the chemicals, therefore indicating bacteria as being solely responsible for the rapid loss of MP and DEP. Several species of bacteria isolated from aufwuchs rapidly transformed MP in dilute nutrient broth. These were identified as *F. aquatile*, *Staphylococcus saprophyticus*, and a bacterium that formed pink colonies but would not transfer well on nutrient agar slants, probably a species of *Flavobacterium*. Most bacteria isolated from aufwuchs could rapidly transform DEP in dilute nutrient broth.

We concluded from the above observations that bacterial transformations of MP and DEP accounted for all of the measurable loss of these chemicals in aufwuchs cultures. Therefore, K_b values derived from our investigations with MP and DEP were based on concentrations of bacteria estimated by standard plate counts.

Effects of carbon sources. The addition of nutrient broth to a final concentration of 0.8 g/liter in blended aufwuchs cultures increased bacterial concentrations approximately 200-fold in 48 h but only increased the rates of MP transformation approximately 3-fold. The addition of glycerol to a final concentration of 1.0 g/liter

increased the bacterial concentration approximately 20-fold in 48 h; no detectable increase in rates of transformation of MP was observed. The same effect was observed with the addition of nutrient broth or glycerol to blended aufwuchs with DEP.

To test for diauxie, K_b for the transformation of DEP was determined by using axenic cultures of *Brevibacterium* sp. in 0, 10, 100, and 1,000 mg of glucose, glycerol, and nutrient broth per liter. The *Brevibacterium* sp. was isolated from aufwuchs. K_b was the same with the additions of glycerol or nutrient broth as it was without any additional nutrient source: $(3.3 \pm 1.0) \times 10^{-11}$ liter per cell per h. K_b for 10 mg of glucose per liter was the same as that for the other carbon sources; however, 100 and 1,000 mg of glucose per liter depressed the rate of DEP transformation one order of magnitude. DEP transformation stopped in the 1,000-mg/liter glucose cultures after the organisms passed the growth phase. *F. aquatile* did not transform MP in a basal salts solution without an additional carbon source, and K_b was the same, $(2.5 \pm 0.29) \times 10^{-13}$ liter per cell per h, in 0.8 and 8.0 g of nutrient broth per liter.

Transformation rate coefficients in aufwuchs. K_b for MP in aufwuchs batch cultures was $(4.5 \pm 11.2) \times 10^{-11}$ liter per cell per h (mean ± standard deviation) and ranged from 9.0×10^{-14} to 8.6×10^{-11} liter per cell per h in 87 cultures. This mean and standard deviation gives a coefficient of variation of 249% for aufwuchs samples from different sites and sampling times. This coefficient of variation can be compared with a coefficient of variation of 33% for variations of K_b between the duplicate samples of blended aufwuchs, showing the large spatial and temporal variations of K_b for MP transformation in aufwuchs. K_b for DEP in batch cultures was $(7.2 \pm 15.4) \times 10^{-10}$ liter per cell per h (mean ± standard deviation) and ranged from 8.6×10^{-12} to 1.1×10^{-8} liter per cell per h in 54 cultures. This mean and standard deviation for DEP transformation gives a coefficient of variation of 214%, compared with coefficient of variation of 32% among the triplicate samples of blended aufwuchs, again showing the large spatial and temporal variations for aufwuchs. Fig. 1 shows the erratic behavior of K_b for MP trans-

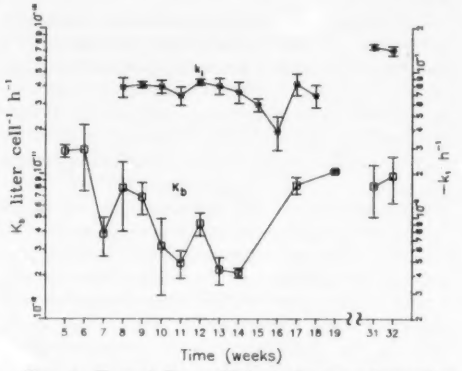

FIG. 1. *K_l and K_b coefficients for transformation of MP in the fermentor, showing the relative stability of K_l compared with K_b. Vertical bars represent twice the standard errors of the means of rates of triplicate batch cultures of aufwuchs for K_b and rates of duplicate water samples for K_l.*

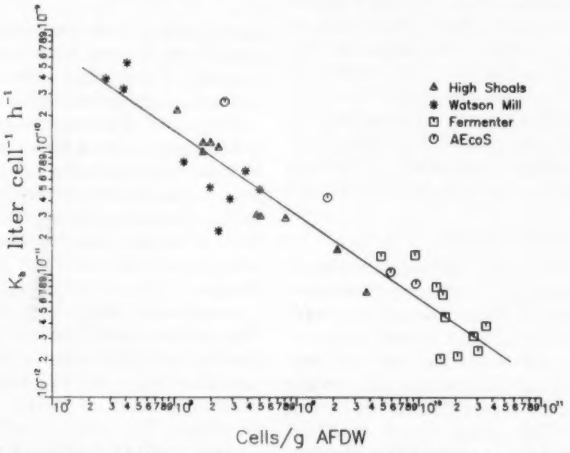

FIG. 2. *K_b for transformation of MP at various concentrations of bacteria in aufwuchs biomass in batch cultures of field and laboratory samples. AFDW was used as a measurement of aufwuchs biomass. K_b decreased as [B] in the aufwuchs biomass increased, thereby giving a relatively constant K_l for a given surface area colonized by aufwuchs, since $K_l = K_b \times [B]$.*

formation in batch cultures of aufwuchs taken from the fermentor over 32 weeks. K_b varied about an order of magnitude, but K_I in the fermentor remained relatively constant. This indicated that K_b was varying in response to fluctuations in concentrations of nontransformer bacteria, because K_I is an indicator of the concentrations of transformer populations, whereas K_b is based on total organism concentrations.

K_b for the transformation of MP decreased as a function of the concentration of bacteria in aufwuchs for the two field sites, AEcoS, and the fermentor (Fig. 2). The same result was obtained for DEP, using aufwuchs samples from the AEcoS.

Variations in proportions and transformation rates of transformer bacteria. To measure variations in proportions of transformer bacteria to total bacteria, colonies on nutrient agar plates were picked and checked for ability to transform MP and DEP in axenic cultures in dilute nutrient broth. Of the 60 colonies picked per plate, only a few transformed MP. With this low percentage of MP transformers, we could not perform experiments with enough isolates to observe statistically significant variations in proportions of transformers. In similar experiments, a much higher rate of DEP transformation was observed: DEP transformers ranged from 20 to 90% of the total bacteria on standard plate counts.

Figure 3 shows that the percentage of DEP transformers correlated with K_b in the CSTRs. The percentage of DEP transformers in aufwuchs samples from five CSTRs was used to calculate a second-order rate coefficient based on transformer bacteria (K_{TB}). Figure 4 shows how K_{TB} varied compared with K_b depending on the concentration of cells in the aufwuchs biomass (as measured by AFDW). The number of DEP transformer bacteria per AFDW unit remained relatively constant over a change in concentration of total cells per AFDW unit of approximately one order of magnitude. The K_{TB}, however, decreased as total cells per AFDW unit increased.

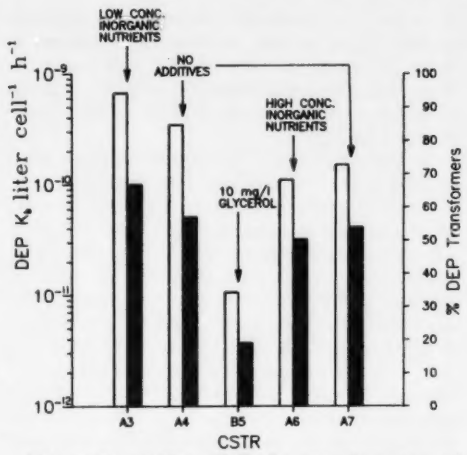

FIG. 3. *Correlation of K_b for transformation of DEP with the percentage of transformer bacteria in batch cultures of aufwuchs samples taken from CSTRs. CSTR 3 had a low concentration of inorganic nutrients; CSTR 4 had no additional nutrients and contained a natural soil sediment; CSTR 5 had 10 mg of glycerol per liter as an organic carbon source; CSTR 6 had 10× the concentration of nitrogen and phosphorus in CSTR 3; and CSTR 7 had no additional nutrients. The percentage of transformer bacteria was determined by picking colonies from standard plate counts and checking the isolates for ability to transform DEP in dilute nutrient broth. Percent transformers represent the number of isolates that transformed DEP (× 100) divided by the total number of colonies picked from a nutrient agar plate. Open bars represent K_b, and closed bars represent the percentage of the total bacteria in aufwuchs that transformed DEP.*

DISCUSSION

Our results with MP and DEP led to two basic conclusions concerning aufwuchs-dominated systems. (i) K_b derived from total plate counts of bacteria varied over at least several orders of magnitude among aufwuchs samples and was related to bacterial concentrations, [B], in the aufwuchs biomass; K_b diminished as [B] in aufwuchs increased. This effect was a result of increases in proportions of nontransformer bacteria as [B] increased and was also a result of rates of transformations by the transformer bacteria decreasing as [B] increased. (ii) K_I for a given surface area was relatively constant compared with K_b over wide ranges in aufwuchs [B] because of the effect described above: K_b decreased as [B] increased ($K_I = K_b \times [B]$).

We expect that our results with MP and DEP will be applicable only to xenobiotic chemicals that are subject to the effect of increased available nutrients selectively increasing nontransformer bacteria disproportionately over transformer bacteria, as we observed with the addition of glycerol or nutrient broth to our aufwuchs cultures. This is most likely the case for the overwhelming majority of xenobiotic pollutants. For certain chemicals that are readily metabolized by virtually all bacteria, increased available nutrients may not disproportionately increase nontransformer bacteria over transformers; therefore, K_b would not decrease as [B] increases, and K_I would not remain constant per unit of surface area.

Our work reveals several directions that could be pursued for developing a rate coefficient for

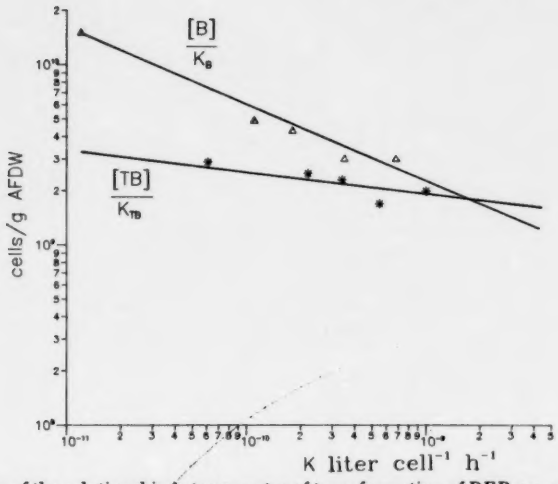

FIG. 4. *Comparison of the relationship between rates of transformation of DEP per cell and the concentration of cells in aufwuchs biomass (AFDW), showing that the rate of DEP transformation per transformer cell decreased as the concentration of total bacteria increased. Symbols: (△) [B] at various rates of transformation per cell, based on K_b; (•) concentration of transformer bacteria in aufwuchs, [TB], at various rates of transformation per transformer cell (K_{TB}).*

microbial transformations of low concentrations of xenobiotic chemicals; such a rate coefficient could have application in predictive modeling for a broad range of aufwuchs-dominated systems. One approach would be to use a mean K_b determined from a random sampling of aufwuchs. The estimated total bacteria associated with aufwuchs in a system would have to be based upon estimates of the submerged surface area bearing aufwuchs communities. The total aufwuchs bacteria multiplied by the mean K_b would result in an estimate of K_l for aufwuchs in the system. A second approach would be to make use of the relative stability of K_l per unit of surface area by determining a rate coefficient derived by dividing K_l by a unit of surface area.

Another approach would be to normalize K_b to some metabolic function of the aufwuchs communities. Because K_b is influenced by the concentration of total bacteria in the aufwuchs and because the bacterial concentration is determined by the nutrient environment, it follows that measurements related to the nutrient environment, such as respiration rates, primary productivity, or perhaps even the transformation rate of a standard xenobiotic chemical could be used to normalize K_b to a specific aquatic system.

Much work remains to be done, particularly pertaining to the basic ecology of transformation processes in mixed microbial communities. We need to know the effects of chemical and physical parameters on these processes and the effects that the activities of microorganisms other than those directly involved in the transformations have on these processes. An understanding of basic microbial phenomena such as adaptation (7) is also essential to developing an accurate method of predicting the transformation rates of xenobiotic pollutants in aquatic systems.

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